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To investigate the expression of Human Immunodeficiency Virus (HIV) genes in human monocytes, a DNA trasfection system was developed and characterized using cultured primary monocytes. Monocytes that were cultured 6-7 days in an adherent monolayer were efficiently recovered and transfectd by electroporation with an expression vector containign the E. coli lacZ gene under control of the cytomegalovirus immediate---early promotor. Successful tranfection was detected by expression of Beta-galactosidase activity and by histochemical staining for Beta-galactorsidase in cells that were allowed to readhere to plastic following transfection. Over 30%of the surviving adherent monocytes expressed the transfected Beta-galactosidase gene. In the same maner, monocytes were transfected with HIV provirus clones pIIIB and pIIIB/PB. The provirus pIIIB/PB differes form pIIIB only in that it contains a small sequence from the 'env' gene of a macrophage tropic HIV-1. Virus derived from pIIIB will not replicate inmonocytes whereas virus derived from pIIIB/PB will. Monocytes tranfected with either provirus DNA expressed hgih levels of p24 antigen within 1 day of transfection, and cell-free supernatants contained virus that was infectious for T cells.

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Transfection of Human Immunodeficiency Virus Type 1 Proviral DNA into Primary Human Monocytes

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To investigate the expression of human immunodeficiency virus (HIV) genes in human monocytes, a DNA transfection system was developed and characterized using cultured primary monocytes. Monocytes that were cultured 6–7 days in an adherent monolayer were efficiently recovered and transfected by electroporation with an expression vector containing the *Escherichia coli lacZ* gene under control of the cytomegalovirus immediate–early promoter. Successful transfection was detected by expression of β-galactosidase activity and by histochemical staining for β-galactosidase in cells that were allowed to readhere to plastic following transfection. Over 30% of the surviving adherent monocytes expressed the transfected β-galactosidase gene. In the same manner, monocytes were transfected with HIV provirus clones pIIIB and pIIIB/PB. The provirus pIIIB/PB differs from pIIIB only in that it contains a small sequence from the *env* gene of a macrophage tropic HIV-1. Virus derived from pIIIB will not replicate in monocytes whereas virus derived from pIIIB/PB will. Monocytes transfected with either provirus DNA expressed high levels of p24 antigen within 1 day of transfection, and cell-free supernatants contained virus that was infectious for T cells. In contrast, only supernatants from pIIIB/PB transfections contained virus capable of infecting monocytes. Thus, proviral DNA of T cell tropic HIV efficiently completes the retroviral life cycle in monocytes in a manner indistinguishable from that of macrophage tropic HIV, and progeny virus retain their T cell tropism.

INTRODUCTION

A variety of techniques have been used to transfer DNA into eukaryotic cells, including calcium phosphate precipitation (1), diethylaminoethyl–dextran transfection (2), liposome-mediated transfection (3), and electroporation (4) (for review see 5). Whereas transformed cell lines can often be easily transfected by one or more of these techniques, primary cells have generally been refractory to transfection with significant efficiency. Recently, methods have been reported that describe the successful transfection of primary T cells (6) and primary B cells (7) by electroporation.

Since human immunodeficiency virus (HIV) infects mononuclear phagocytes (monocytes and macrophages) *in vitro*, and since tissue macrophages have been found to harbor HIV *in vivo* (8–10), it would be advantageous to study HIV gene expression directly in primary human monocytes. However, not all HIV-1 isolates will replicate in monocytes. Indeed, HIV-1 isolates can be divided into two groups depending upon

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the type of cells that they infect *in vitro*. Macrophage tropic virus infects both primary monocytes and T cells; T cell tropic virus infects primary T cells but not primary monocytes. Macrophage tropic isolates are prevalent early in HIV infection, whereas the frequency of T cell tropic virus increases with disease progression and may be causally associated with HIV-1 pathogenesis (11, 12).

Analysis of hybrid virions derived from macrophage and T cell tropic strains of HIV-1 has shown that the principal element for control of macrophage tropism resides within a 20 amino acid sequence in the V3 loop of gp120 that is distinct from the CD4-binding sequence (13–16). This suggests that the mechanism for restricted cell tropism of HIV-1 involves the internalization of viral particles after gp120–CD4 binding. Presumably, once proviral DNA is made from either macrophage or T cell tropic HIV-1, replication should proceed in human monocytes. To test this hypothesis, an efficient DNA transfection system for primary human monocytes was developed. Monocytes transfected with either macrophage or T cell tropic HIV-1 DNA produced infectious HIV-1, but progeny virus retained the cell tropism of the original DNA provirus.

MATERIALS AND METHODS

Cells. Peripheral blood mononuclear cells were obtained from HIV and Hepatitis B seronegative blood donors following leukapheresis and separation over Ficoll–Hypaque gradients. Monocytes were purified by countercurrent centrifugal elutriation (90–95% pure) and cultured as adherent cell monolayers in RPMI 1640 (GIBCO, Grand Island, NY) with 10% fetal bovine serum (Hyclone, Logan, UT), supplemented with glutamine (20 m*M*), gentamicin (50 μg/ml), and 1000 U/ml recombinant human macrophage colony stimulating factor (M-CSF; a gift from Chiron Corp., Emeryville, CA). Freshly elutriated cells were allowed to adhere in serum-free medium for approximately 1 hr before the addition of an equal volume of complete medium. Half of the medium was changed the next day and every second day afterward. Peripheral blood lymphocytes (PBL), approximately 95% pure, were obtained from elutriation of mononuclear cells, cultured for 3 days in RPMI 1640 supplemented with 15% fetal bovine serum, 2 m*M* glutamine, 50 μg/ml gentamicin, IL-2 (Advanced Biotechnologies, Inc., Columbia, MD), and phytohemagglutinin (PHA, 1 μg/ml) before being infected.

Plasmids. Plasmids pCMVβ and pNASSβ, with the *Escherichia coli lacZ* gene under the control of the cytomegalovirus immediate-early promoter or without a promoter, respectively, were obtained from Clonetech (Palo Alto, CA). The HIV-1 provirus clones pIIIB and pIIIB/PB were a gift from Dr. Bryan Cullen (13) and were propagated in *E. coli* strain MC1061.

β-Galactosidase assays. Assays for β-galactosidase activity were done essentially as described by Miller (17), but adapted for 96-well plates. Cells were resuspended in a final volume of 50 μl of phosphate-buffered saline (PBS), freeze thawed three times, and samples were clarified with a brief spin in a microfuge. Samples were diluted in PBS, as were β-galactosidase standards, and 20 μl was used per well. Assays were stopped at 2 hr and the A_{405} was determined with a microplate reader.

p24 Assays. Levels of p24 antigen in cell-free supernatants were determined by ELISA (Coulter Immunology, Hialeah, FL).

Histochemical staining for β-galactosidase. Monocytes were stained for expression of β-galactosidase essentially as described by Dannenberg and Suga (18). Briefly,

monolayers were washed with PBS and fixed with 2% glutaraldehyde in PBS for 5 min, followed by 4% formaldehyde for 5 min. After washing again with PBS, the cells were incubated for 1 hr with stain [1.0 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 0.01% sodium deoxycholate, 0.02% NP-40, 5 m*M* potassium ferricyanide, 5 m*M* potassium ferrocyanide, and 2 m*M* $MgCl_2$ in PBS]. After staining, the cells were washed with PBS and counterstained with Mayer's hematoxylin solution for 10 min and washed with PBS.

Transfections. Monocytes were collected for transfection after 6 or 7 days of culture in 6-well plates. Half of the media was gently removed and discarded. The remaining media and loosely adherent cells were transferred to centrifuge tubes. The monolayers were washed once with 1 ml per well of cold PBS (without calcium and magnesium), and the wash was saved. The monolayers were then incubated with 2 ml of cold PBS per well for 5 min at 4°C and then the cells were gently scraped into the PBS and pooled along with a final wash of the plates (1 ml per well). The pooled monocytes were collected by centrifugation at 1000 rpm for 10 min, washed one time in cold PBS, and counted. After a final centrifugation, the cells were resuspended in PBS at a concentration of 4×10^7 cells/ml and 0.25 ml (1×10^7 cells) was aliquoted into 0.4-cm electroporation cuvettes. DNA was added to each sample and the mixture was incubated on ice for 5 min before electroporation with a Bio-Rad Gene Pulser (Bio-Rad, Richmond, CA) under the indicated conditions. After transfection, the cuvettes were returned to ice for 5 min. The cells were resuspended in serum-free media and allowed to readhere to tissue-culture plates for 1 hr before the addition of an equal volume of complete monocyte media. For analysis of β-galactosidase activity, the cells were scraped into the media at 24 hr after transfection, collected by centrifugation, washed one time with PBS, and resuspended in a final volume of 50 μl. For analysis of p24 antigen, cell-free supernatants were assayed at 24 hr after transfection.

RESULTS AND DISCUSSION

Transfection of human monocytes. Monocytes that had been cultured for 6 to 7 days were collected for electroporation by washing and scraping in cold PBS as described under Materials and Methods. Approximately 75% of the original number of monocytes were recovered in this manner and were >95% viable, as determined by trypan blue exclusion. When resuspended in serum-free media, the cells adhered to plastic and formed a monolayer indistinguishable from the original monolayer (Fig. 1). Thus, culture and recovery of monocytes prior to electroporation had little or no effect on cell viability or ability to attach to plastic substrates.

To optimize electroporation of monocytes, the reporter plasmids pCMVβ and pNASSβ were used to transfect the collected cells. Each plasmid contains the *E. coli lacZ* gene, but only pCMVβ has a eukaryotic promoter to direct RNA synthesis and, thus, pNASSβ served as a transfection control for background β-galactosidase (β-Gal) activity. Lysates of cells transfected under various conditions were analyzed for β-Gal activity at 24 hr post-transfection. A capacitance setting of 960 μF resulted in the highest level of β-Gal activity, as did a voltage setting of 0.25 kV (Fig. 2). At these electroporation settings, 25 μg of DNA and a cell concentration of 4×10^7 cells/ml were optimal (Fig. 3). The β-Gal activity measured under optimal conditions at 24 hr post-transfection was approximately 1.5 times that measured at 48 hr (data not shown).

There was a fairly small range for each electroporation parameter that could be used for successful transfection. In general, electroporation conditions that yielded an

FIG. 1. Recovery and replating of cultured primary monocytes. (A) Human monocytes were cultured for 6 days as described under Materials and Methods. Cells were fixed with glutaraldehyde and formaldehyde and counterstained with Mayer's hematoxylin solution for 10 min. Magnification was 20×. (B) Monocytes that had been cultured for 6 days were collected by scraping into cold PBS, washed, and allowed to readhere. Cells were fixed and counterstained as in A.

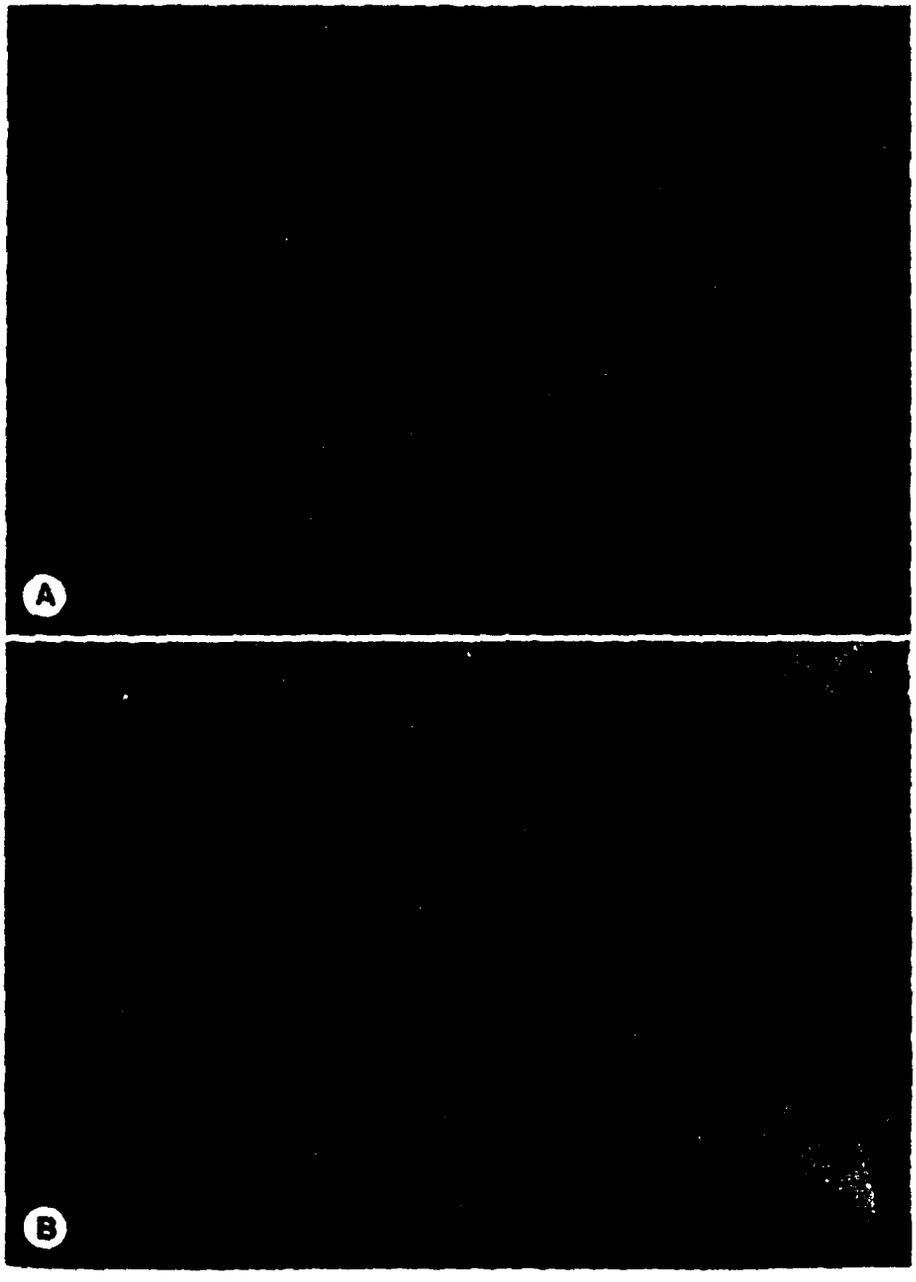

FIG. 4. Histochemical staining of transfected monocytes for β-galactosidase. Monocytes were transfected with either pNASSβ (A) or pCMVβ (B) and, at 24 hr post-transfection, fixed and stained as described under Materials and Methods.

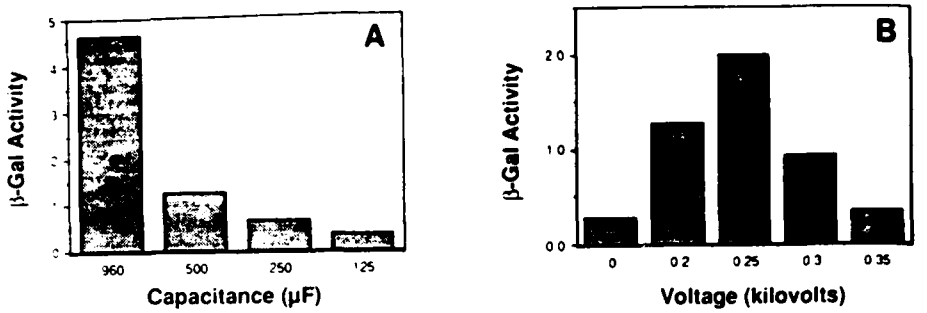

FIG. 2. Transfection of human monocytes. (A) Monocytes were electroporated at 0.25 kV and the indicated capacitance settings. At 24 hr postinfection, cells were lysed and β-galactosidase activity was measured as described under Materials and Methods. (B) Expression of β-galactosidase activity in lysates of monocytes transfected at 960 μF and increasing voltage settings.

increase in the number of surviving cells resulted in a decrease in β-Gal activity. Similar observations have been previously reported (for review see 5).

The expression of *lacZ* from pCMVβ-transfected cells was profoundly affected by the length of time that monocytes were cultured before transfection. Under optimal conditions established for monocytes cultured for 6 or 7 days, no β-galactosidase activity was detected in freshly isolated monocytes or cells cultured as an adherent monolayer for 1 or 2 days when transfected with pCMVβ. Monocytes that were cultured for 4, 6, or 7 days before transfection with pCMVβ expressed equivalent levels of β-galactosidase activity (data not shown). It is not clear whether these differences in β-galactosidase activity reflect the activity of the CMV promoter at different stages of monocyte differentiation or are due to changes in transfection efficiency induced by cell differentiation.

Frequency and characterization of transfected cells. The use of *lacZ* as a marker gene permitted determination of transfection efficiency by histochemical staining of transfected cells. At 24 hr after transfection, the monolayers were washed with PBS and fixed. Following fixation, the cells were incubated for 1 hr with X-Gal stain,

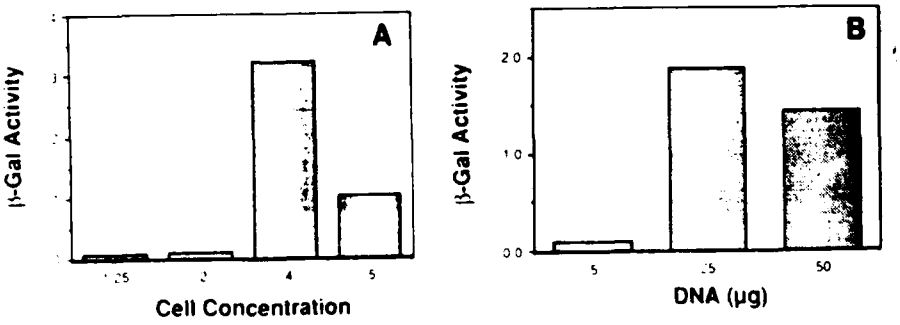

FIG. 3. Effect of DNA concentration and cell concentration on transfection. (A) 1×10^7 monocytes were transfected at 0.25 kV and 960 μF at 1.25×10^7, 2×10^7, 4×10^7, and 5×10^7 cells/ml. β-galactosidase activity was measured at 24 hr postinfection. (B) Monocytes were transfected at 0.25 kV and 960 μF with the indicated amounts of pCMVβ DNA.

washed, and counterstained. No pNASSβ-transfected cells stained blue for β-galactosidase (Fig. 4A). In contrast, over 30% (32 ± 6) of the pCMVβ-transfected cells appeared dark blue at this time (Fig. 4B). To determine the recovery of cells after transfection, duplicate cultures of transfected monocytes were collected by scraping into cold PBS and counted. Approximately 13% of the original number of cells were recovered; >75% of the recovered cells were viable. These cells were 97% monocytes as determined by cell morphology on Wright–Giemsa-stained cytospins.

Recently, two separate reports have described successful transfection of primary monocytes. One of these reported between 1 in 500 and 1 in 1000 cells expressing β-galactosidase after transfection (19); the other did not report observed transfection efficiencies (20). In both of these systems, monocytes were isolated by adherence rather than elutriation. It is not known whether different isolation and culture procedures might affect the efficiency of monocyte transfection.

Transfection of monocytes with HIV provirus DNA To determine whether there was any restriction on the replication of T cell tropic HIV-1 in monocytes that was independent of the *env* gene, monocytes were transfected with the provirus clones pIIIB and pIIIB/PB (13). Plasmid IIIB is a full-length, replication-competent clone derived from the HTLV-IIIB isolate of HIV-1. Virus derived from this clone will not replicate in monocytes. Plasmid IIIB/PB, however, has a sequence from the envelope region of the macrophage tropic virus BaL substituted for the corresponding region in pIIIB, and virus derived from this clone replicates well in primary monocytes. When these provirus DNAs were transfected into monocytes, p24 antigen was detected at 24 hr post-transfection (Table 1). The p24 antigen levels at 24 hr after transfection with the two proviruses were not significantly different ($P = 0.38$).

The production of viable virus progeny from transfected monocytes was determined by adding the cell-free supernatants from pIIIB and pIIIB/PB transfections to cultures of uninfected monocytes or PBL. Supernatants from both pIIIB and pIIIB/PB transfected monocytes contained progeny virus able to infect PBL. PBL were cultured as described under Materials and Methods before being infected with cell-free supernatants from pIIIB and pIIIB/PB transfections. After a 3-hr incubation, cells were washed and resuspended in fresh media. Culture samples were collected at this time and at 3 and 9 days after infection. p24 antigen was undetectable immediately after infection. Levels of p24 antigen in these infected PBL cultures were 8 (pIIIB) and 45 (pIIIB/PB) pg/ml at 3 days after infection and 125 (pIIIB) and 300 (pIIIB/PB) pg/ml at 9 days after infection. In contrast, only supernatants from pIIIB/PB-transfected monocytes were

TABLE 1

Transfection of Proviral DNA

Plasmid DNA	p24 (pg/ml)
Mock transfection	<8
pNASSβ	<8
pIIIB	270 ± 20
pIIIB/PB	370 ± 100

Note. Monocytes were transfected as described in the text with 25 μg of plasmid DNA. Supernatant was assayed for p24 antigen (Coulter Immunology) at 1 day post-transfection. p24 values are expressed as the mean ± standard error of three experiments.

able to infect other monocytes (Fig. 5). Monocyte monolayers were infected for 2 hr, washed two times, and given fresh media. Samples for p24 antigen assay were taken at this time and every 2 days thereafter. In cultures infected with pIIIB/PB supernatants, p24 antigen was detected by 6 days and continued to increase for at least 14 days after infection. Progeny virus from pIIIB-transfected monocytes, while fully infectious for PBL, showed no signs of replication in monocytes. No p24 antigen was detected at any time through 2 weeks after infection.

As shown previously in several reports (13–16), substitution of a small region of the envelope from a virus strain that is capable of replicating in monocytes permits replication in monocytes. By DNA transfection of such proviral clones, we have shown that there is no significant barrier to gene expression or replication of T cell tropic HIV-1 in monocytes past the initial events associated with virus entry into the cell. To the contrary, when IIIB DNA was introduced directly into monocytes by transfection, viable progeny virus retaining its original T cell tropism was produced. This suggests that the only impediment to IIIB expression and replication in human monocytes is in entry into these cells. The ability to separate HIV entry into monocytes from proviral gene expression should permit a careful evaluation of the contribution of individual gene products to virus replication.

Just as important, an efficient transfection system for primary monocytes will also permit studies on the regulation of HIV gene expression in monocytes. Previously, continuous myeloid cell lines such as U937 and THP-1 were transfected as model systems to investigate the activation of HIV gene expression by NF-κB induction and LPS stimulation (21, 22). Whereas NF-κB binding activity is induced in U937 and THP-1 cells by PHA, PMA, or LPS, this binding activity is constitutively present in primary monocytes. In contrast to LPS stimulation of HIV gene expression in U937 and THP-1 cells, LPS decreases HIV gene expression in primary monocytes (19). The ability to separate HIV entry into monocytes from proviral gene expression should permit analysis of the contribution of individual HIV genes and their products to HIV replication in primary monocytes.

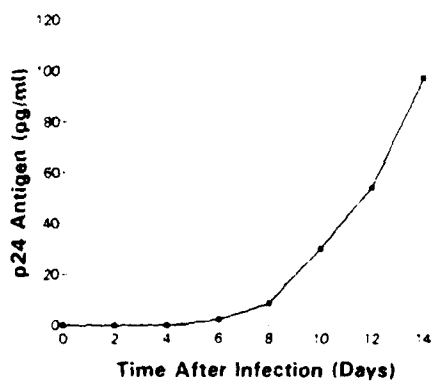

FIG. 5. Infection of monocytes with supernatant from pIIIB/PB-transfected monocytes. Monocytes (1×10^7) were transfected with pIIIB/PB or pIIIB, and the supernatants were collected at 24 hr and filtered (0.4 μm). Half of this was used to infect $\sim 1.5 \times 10^6$ cultured monocytes. Samples were taken every 2 days for p24 assay.

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